IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : Leu-Fen H. Lin, Franklin D. Collins, Daniel H. Doherty, Jack Lile, and Susan Bektesh

Serial No.: Not Yet Assigned — Group Art Unit No.: TBD

Filed: Concurrently herewith — Examiner: Not Yet Assigned

For: GLIAL DERIVED NEUROTROPHIC FACTOR

Docket No.: S225-M

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Prior to examination, please amend the referenced application as follows:

In the Specification

On page 1, please replace the present title, GLIAL DERIVED NEUROTROPHIC FACTOR with GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR.

On page 1, after the title, please insert the following 35 U.S.C. § 120 statement:

CROSS REFERENCE TO RELATED APPLICATION(S)

This application is a divisional of application Serial No. 08/452,229, filed May 26, 1995, which is a which is a divisional of 08/182,183; filed May 23, 1994, which is a 35 U.S.C. §371 filing of PCT/US92/07888, filed September 17, 1992; which is a continuation-in-part of 07/855,413, filed March 19, 1992, now abandoned; which is a continuation-in-part of 07/788,423, filed November 6, 1991, now abandoned; which is a continuation-in-part of 07/774,109, filed on October 8, 1991, now abandoned; which is a continuation-in-part of

EXPRESS MAIL CERTIFICATE

"Express Mail" mail labeling number EL360689529US Date of Deposit November 13, 2001

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box Patent Application, Assistant Commissioner for Patents, Washington, D.C. 20231

Lynne Buchsbaum — Date

Lynne Buchsbaum — Signature

07/764,685, filed September 20, 1991, now abandoned; which are hereby incorporated by reference.

Please replace the paragraph on Page 1, lines 4 to 15 with the following new paragraph:

The present invention relates to neurotrophic factors and glial cell line-derived neurotrophic factor (GDNF) in particular. Also included within this invention are processes for purification of GDNF from natural sources and processes for cloning rat and human genes encoding GDNF, as well as the nucleic acid sequence of the rat and human genes that encode GDNF. The GDNF gene has been subcloned into an expression vector, and the vector used to express biologically active GDNF. In addition, this invention includes the use of GDNF for preventing and treating nerve damage and nerve related diseases such as Parkinson's disease.

Please replace the paragraph on Page 7, lines 21 to 28 with the following new paragraph:

This invention relates to and claims substantially purified glial cell line-derived neurotrophic factor (GDNF). In one embodiment of this invention, substantially purified GDNF is obtained having a specific activity at least about 24,000 times greater than the specific activity of B49 conditioned medium. The substantially purified GDNF has a specific activity of at least about 12,000 TU/μg.

Please replace the paragraph on Page 8, lines 1 to 7 with the following new paragraph:

The amino acid sequence of mature and "pre-pro" forms of rat GDNF is as set forth in Figs. 13 and 14 (SEQ ID NO:3 and SEQ ID NO:4). The amino acid sequence of mature human GDNF is as set forth in the underlined portion of Fig. 19 (SEQ ID NO:6). The amino acid sequence of the pre-pro form of human GDNF is set forth in Figures 19 (SEQ ID NO:5) and 22 (SEQ ID NO:8).

Please replace the paragraph on Page 9, lines 28 to 37 and Page 10, lines1 to 2 with the following new paragraph:

This invention includes substantially purified antibodies that recognize GDNF. Also included is a method for preventing or treating nerve damage which comprises implanting cells that secrete glial cell line-derived neurotrophic factor into the body of patients in need thereof. Finally, the present invention includes a device for preventing or treating nerve damage by implantation into a patient comprising a semipermeable membrane, and a cell that secretes

GDNF encapsulated within said membrane and said membrane being permeable to GDNF and impermeable to factors from the patient detrimental to the cells.

Please replace the paragraph on Page 11, lines 5 to 12 with the following new paragraph:

Figure 8 (SEQ ID NO:1) describes the amino-terminal amino acid sequence obtained from purified GDNF. The empty parenthesis indicates a position where the amino acid could not be determined using the sequencing technique employed. Where residues are given in parentheses, there was some uncertainty as to the identification of that residue. The complete correct amino-terminal amino acid sequence is shown in Figure 19 (SEQ ID NO: 4) below.

Please replace the paragraph on Page 11, lines 25 to 26 with the following new paragraph:

Figure 12 (SEQ ID NO:2) describes an internal amino acid sequence obtained from purified GDNF.

Please replace the paragraph on Page 11, lines 28 to 34 with the following new paragraph:

Figure 13 (SEQ ID NO:3) depicts the nucleic acid sequence obtained for rat GDNF derived from a B49 cell library cDNA clone AZapII76.1. SEQ ID NO:4 depicts the inferred amino acid sequence for rat GDNF. In Figure 13, the nucleic acid sequence coding for mature GDNF is underlined. The amino-terminal sequence of the most preferred pre-pro form of GDNF is marked with an *.

Please replace the paragraph on Page 11, lines 36 to 37 with the following new paragraph:

Figure 14 (SEQ ID NO:4) depicts the inferred amino acid sequence of mature rat GDNF.

Please replace the paragraph on Page 13, lines 17 to 23 with the following new paragraph:

Figure 19 (SEQ ID NO:5) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2C below, including the entire portion of the gene encoding for mature human GDNF. SEQ ID NO:6 depicts the inferred amino acid sequence for mature human GDNF. In Figure 19, the amino acid sequence for mature human GDNF is underlined.

Please replace the paragraph on Page 14, lines 18 to 27 with the following new paragraph:

Figure 22 (SEQ ID NO:8) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2D below, including the coding sequence of amino acids of 1-50 of human pre-pro GDNF. Also depicted is the inferred amino acid sequence for the first 50 amino acids of human pre-pro GDNF. This information, in conjunction with coding sequence information given in Figure 19, provides the full coding sequence for human pre-pro GDNF, and the inferred amino acid sequence for the human pre-pro GDNF protein.

Please replace the paragraph on Page 16, lines 14 to 24 with the following new paragraph:

Prior to this invention, GDNF had not been identified as a discrete biologically active substance and had not existed in a substantially pure form. As described herein, a detailed description of GDNF is provided, along with a description of: its physical, chemical and biological characteristics; its utility; how to make it; useful compositions containing it; nucleic acid sequences coding for it; vectors containing such nucleic acid sequences; host cells transformed by such vector; recombinant techniques for its production; and other aspects of the invention.

Please replace the paragraph on Page 18, lines 29 to 33 with the following new paragraph:

Probes were prepared based on the nucleic acid sequence of rat GDNF in order to clone the genomic DNA gene coding for human GDNF. The human gene encoding mature GDNF, and the amino acid sequence of human mature GDNF are given in Figure 19 (SEQ ID NOS:5 and 6, respectively).

Please replace the paragraph on Page 19, lines 17 to 26 with the following new paragraph:

Throughout this specification, any reference to glial cell line-derived neurotrophic factor should be construed to refer to neurotrophic factors of any origin which are substantially homologous to and which are biologically equivalent to the GDNF characterized and described herein. The degree of homology between the rat and human protein is about 93% and all mammalian GDNF will have a similarly high degree of homology. Such GDNFs may exist as dimers in their biologically active form.

Please insert the following paragraph on Page 20, line 22 after "reference.":

In particular, Dayhoff describes that "[i]n practice, two related proteins may be aligned with the insertion of an average of 3 or 4 gaps in a length of 100 residues. About 20% of the aligned amino acids are identical. Under these conditions, the statistical conclusion of common ancestry can be drawn with great confidence. Common ancestry may exist even though it cannot be proved from the comparison of two sequences. The use of additional evidence, such as the correspondence of the active sites, the comparisons of many related sequences with one new one, and the nature of the three-dimensional structures, will eventually permit the inference of relationships of even more remotely related structures."

Please replace the paragraph on Page 26, lines 11 to 20 with the following new paragraph:

The procedure for obtaining the nucleotide sequence of the cDNA clone contained in λZapII76.1 is given in Example 2B. below. The nucleotide sequence of the first 877 base pairs of the 5' end of the cDNA clone was determined, and is shown in Figure 13 (SEQ ID NO:3). In Figure 13, the clone shown contains an open reading frame (ORF) of 227 amino acids that includes the amino-terminus of purified GDNF and is consistent with the sequence for an internal peptide obtained by cleavage of purified GDNF.

Please replace the paragraph on Page 26, lines 21 to 38 and Page 27, lines 1 to 9 with the following new paragraph:

The inferred amino acid sequence given in Figure 14 (SEQ ID NO:4) shows the amino acid sequence for the "mature GDNF". By "mature GDNF", is meant the sequence of the purified GDNF obtained from the B49 conditioned medium. Of course, the purified GDNF may exist as a dimer or other multimer and may be glycosylated or chemically modified in other ways. Mature GDNF may be truncated at the carboxyl terminus, in particular by proteolytic processing of the lys-arg residues 6 and 5 residues from the carboxyl terminal end. Examination of the nucleic acid sequence of the λZapII76.1 rat clone as shown in Fig. 13 (SEQ ID NO:3) suggests that GDNF is initially translated as pre-pro-GDNF polypeptide and that proteolytic processing of the signal sequence and the "pro" portion of this molecule result in purified GDNF having the same mature sequence as that obtained from B49 conditioned medium. It is postulated, that the pre-pro-GDNF polypeptide begins at the first ATG --methionine encoding-- codon at the 5' end of the clone (position 50 in Figure 13). The present invention includes, therefore, any and all

pre-pro GDNF polypeptides that may be translated from the gene shown in Figure 13, as well as any and all pre-pro GDNF polypeptides translated from a more complete clone that may be easily obtained by one of skill in the art using standard laboratory procedures and the clone described herein.

Please replace the paragraph on Page 27, lines 10 to 24 with the following new paragraph:

Review of the rat nucleic acid sequence given in Fig. 13 (SEQ ID NO:3) shows that the predicted amino acid sequence located between positions 518 and 538 is Asp-Lys-Ile-Leu-Lys-Asn-Leu which is consistent with the amino acid sequence determined for a peptide derived from purified mature GDNF by the process described in the section on internal sequence in Example 1 below. A TGA stop codon at position 706 terminates the ORF. The predicted length of the purified GDNF is thus 134 amino acid residues, and the predicted molecular weight of this polypeptide is 14,931. Two potential N-linked glycosylation sites occur at positions 425 and 533. Glycosylation at either or both of these sites would increase the molecular weight of the molecule.

Please replace the paragraph on Page 30, lines 11 to 27 with the following new paragraph:

Specific nucleic acid sequences can be modified by those of skill in the art. Therefore, this invention also includes all nucleic acid sequences which encode for the amino acid sequences for mature rat and mature human GDNF as set forth in Figures 14 (SEQ ID NO:4) and 19 (SEQ ID NO:6), and pre-pro rat GDNF as set forth in Figure 13 (SEQ ID NO:3) and for pre-pro human GDNF as set forth in Figures 19 (SEQ ID NO:5) and 22 (SEQ ID NO:8). The present invention also incorporates nucleic acid sequences which will hybridize with all such nucleic acid sequences -- or the complements of the nucleic acid sequences where appropriate -- and encode for a polypeptide having dopaminergic activity. The present invention also includes nucleic acid sequences which encode for polypeptides that have dopaminergic activity and that are recognized by antibodies that bind to GDNF.

Please replace the paragraph on Page 36, lines 23 to 37 and Page 37, lines 1 to 16 with the following new paragraph:

An alternative method for identifying GDNF family members involves use of the polymerase chain reaction (PCR) to amplify sequences from GDNF family members followed by

cloning and analysis of amplified sequences. Degenerate (or nondegenerate) oligonucleotide primers for PCR may be synthesized based on the sequence of GDNF. Given the conservation of cysteine location and the conservation of amino acid sequences in the immediate vicinity for the cysteine residues that is observed for the NGF family, the regions around the cysteines in mature GDNF represent obvious candidates for primer synthesis but a variety of other primers could also be chosen from both the mature and pre-pro-portions of the protein. PCR reactions may be performed under conditions of reduced annealing temperature which would allow amplification of not only the GDNF sequence but the sequences of any GDNF family members. See, Innis et al. 1990 PCR Protocols: A Guide to Methods and Applications, Academic Press. The products of such PCR reactions may be size selected by gel electrophoresis, cloned into an appropriate vector and the cloned DNA sequenced to identify GDNF family members. Alternatively, the clones may first be screened by hybridization to a probe specific for GDNF under conditions of high stringency to identify GDNF clones. Any clones that fail to hybridize to GDNF under high stringency would then be sequenced or such clones could be hybridized to a GDNF probe under conditions of reduced stringency and any clone that did hybridize to the GDNF probe under these conditions would then be sequenced.

Please replace the paragraph on Page 45, lines 25 to 37 and Page 46, lines 1 to 9 with the following new paragraph:

The functional status of dopaminergic neurons may be assayed in these cultures by measuring dopamine uptake through specific "scavenger" transporters in the dopaminergic neurons and by counting the number of neurons positive for the dopamine synthetic enzyme tyrosine hydroxylase using immunohistochemistry. The possibility of significant contamination of the cultures with the noradrenergic neurons, which can also transport dopamine and also contain tyrosine hydroxylase, was ruled out by careful dissection and by demonstrating that the dopamine transporters have the pharmacological profile characteristic of dopaminergic, rather than noradrenergic, neurons. Dopamine uptake in these cultures is inhibited by GBR12909, an inhibitor of the monoamine transporter on dopaminergic neurons, with an ED_{50} of 20nM. In contrast, at least 300-fold more desipramine, an inhibitor of monoamine transporter or noradrenergic neurons, is required to inhibit dopamine uptake in their cultures. These values are those that have been reported for the monoamine transporter in dopaminergic neurons.

Please replace the paragraph on Page 51, lines 1 to 37 and Page 52, lines 1 to 3 with the following new paragraph:

Internal sequence: GDNF preparation after step 3 of the purification described above was used as the starting material to obtain internal sequence. Fractions 5 and 6 in Figure 3 were pooled into a siliconized microfuge tube containing 9 µl of 0.4% Tween and concentrated to 40 µl on a speed vac. Added to the sample were 160 µl of 1% NH_4HCO_3 containing 2.5 M guanidine hydrochloride and 1 µg of trypsin, and the sample was incubated overnight at 37°C. The mixture was acidified with 20 µl of 25% TFA, concentrated to about 150 µl on a speed vac, and peptides were separated on a narrow bore Aquapore RP-300 C8 reverse phase HPLC column (Brownlee column), 2.1 x 220 mm, and eluted with an H_2O/0.1% TFA:80% acetonitrile/0.085% TFA gradient. Peptide containing fractions were collected manually into siliconized microfuge tubes based on the absorption at 214 nm. Figure 9 shows the results of such chromatography. Sequence of peak 10 in Figure 9 was determined to be identical to the first 13 amino acid residues of the amino-terminal sequence of the untreated protein shown in Figure 8 (SEQ ID NO:1). Peak 37 in Figure 9 was further treated with CNBr. The sample was concentrated to 20 µl on a speed vac. Added to the sample was 70 µl of 90% formic acid and 5 mg of CNBr, and the sample was incubated in the dark overnight at room temperature. This mixture was concentrated to 20 µl on a speed vac, diluted with 100 µl of 0.1% TFA and separated on reverse phase HPLC as described above. Figure 10 shows the results of such chromatography. Peak 1 in Figure 10 was concentrated to 20 µl in the presence of 5 µl of 0.4% Tween 20 on a speed vac. Added to the sample was 100 µl of 1% NH_4HCO_3 and 5 µl of 50 mM dithiothreitol and the sample was incubated at room temperature for an hour. The mixture was acidified with 10 µl of 25% TFA, concentrated to 100 µl on a speed vac and separated on reverse phase HPLC as above. Figure 11 shows the results of such chromatography. Both peaks 33 and 34 in Figure 11 gave an identical sequence (Figure 12) (SEQ ID NO:2).

Please replace the paragraph on Page 64, lines 27 to 37 and Page 65, lines 1 to 11 with the following new paragraph:

Six λFIX II clones from a human genomic library were identified by hybridization with a rat GDNF probe and plaque-purified to homogeneity (see above). Lysates of each phage were prepared by the method of Sambrook et al. (Molecular Cloning: A Laboratory Manual; 1989). DNA was prepared from these clones by the following procedure: DNAase I (Pharmacia) and

RNAase A (Sigma) was added to 5 ml of each culture to give a final concentration of 1 μg/ml. The solution was incubated at 37°C for 1 hour. Then 5 ml of 20% polyethylene glycol (Sigma), 2M NaCl, was added and the solution was incubated at 0°C for 1 hour. The λphages were pelleted by centrifugation at 12,000 x g for 10 min. The phage pellet was resuspended in 250 μl of TE (10mM TRIS, pH 7.4, 1mM EDTA) and sequentially extracted with an equal volume of: a. chloroform; b. phenol; c. a 1:1 mixture of chloroform and phenol; and d. chloroform. Ammonium acetate was added to give a final concentration of .25 M and the DNA was precipitated by the addition of 2 volumes of ethanol and centrifugation at 10,000 x g. The DNA pellet was resuspended in TE.

Please replace the paragraph on Page 66, lines 35 to 37 and Page 67, lines 1 to 10 with the following new paragraph:

To obtain the complete human pre-proGDNF sequence, a radiolabeled hybridization probe may be made based on the sequence of human GDNF already obtained, and this probe may be used to screen human cDNA libraries. Because cDNAs are copies of the processed mRNA, the introns are not present and the sequence of the complete coding sequence can be obtained. Alternatively, now that the position of the intron relative to the coding sequence in known, a hybridization probe that is specific for sequences upstream of the intron can be made from the rat DNA clone and this probe can be used to screen a genomic library for clones that contain the 5' exon(s).

Please replace the paragraph on Page 67, lines 12 to 23 with the following new paragraph:

D. Nucleotide Sequence Encoding the First 50 Amino Acids of Human Pre-proGDNF

As detailed in Example 2C, there is an intron that splits the nucleotide sequence corresponding to amino acid 51 of human pre-proGDNF (i.e. between the T and CA forming the codon for the serine residue at position 51). In order to obtain the sequence of this portion of the molecule, a human genomic library was screened with a probe derived from the amino-terminal coding sequence of rat pre-proGDNF and one hybridizing clone was sequenced and shown to contain the coding sequence of amino acids 1 through 50 of human pre-proGDNF as shown in Figure 22 (SEQ ID NO:8). SEQ ID NO:25 and SEQ ID NO:26 present nucleotide and amino acid sequences, respectively, for a composite pre-pro sequence as depicted in Figures 22 and 19

as well as SEQ ID NOS:8 and 5. A pre-pro form of human glial cell line-derived neurotrophic factor polypeptide is set forth in SEQ ID NO:26 amino acid residues 10 through 220.

Please replace the paragraph on Page 68, lines 13 to 37 and Page 69, lines 1 to 4 with the following new paragraph:

The cloned DNAs of these recombinant phages were analyzed by Southern blot hybridization. An approximately 1000 bp AluI fragment was found to hybridize to the screening probe and was subcloned into SmaI-digested pBluescript SK- to produce pBSSK-λ3AluI. This cloned AluI fragment was further subcloned to facilitate sequencing of the relevant DNA segment. Purified pBSSK-λ3AluI DNA was digested with a series of restriction enzymes that cleave the vector only once (within the polylinker region) and the digestion products were analyzed by agarose gel electrophoresis. Two restriction enzymes (PstI and SacII) were thus identified that cleaved once within the cloned DNA. Figure 22 shows a map of SacII and PstI sites. Southern blots revealed that the region of the cloned segment that hybridized to the screening probe was located between the SacII and PstI sites of the cloned AluI segment. Therefore, for sequencing, two deletion derivatives of pBBSK- λ3AluI were constructed. In one instance the small, about 300 bp, PstI fragment was deleted as follows: The plasmid was digested with PstI and the digest was ligated and transformed into E. coli. Transformants were screened for those lacking the small PstI fragment. In parallel, the 300 bp SacII fragment was similarly deleted from pBSSK-λ3AluI to produce a second deletion derivative. These two deletion plasmids were used as templates for sequencing reactions. Sequencing was carried out as described in Example 2B.

Please replace the paragraph on Page 78, lines 16 to 37 and Page 79, lines 1 to 10 with the following new paragraph:

The reaction conditions for the amplification of the human GDNF are as follows: the total reaction volume was 100μl and contained 1 ng of a λDNA clone containing the human GDNF gene, 20 pmoles each of PD3 and PD4, 20 mM Tris-HCl pH8.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. The reaction mixture was heated to 95°C for 5 minutes, cooled to 44°C, and 2 units of Pfu DNA polymerase (Stratagene) were added. The reaction consisted of 30 cycles of: 72°C for 1½ minutes, 95°C for 1 minute, and 44°C for 1½ minutes. At the end of the reaction, $MgCl_2$ was added to a final concentration of 10 mM. 5 units

of DNA polymerase I large (Klenow) fragment (Promega) were added, and the reaction incubated at 37°C for 10 minutes. Then 10µl of 3M NaAc and 220µl of EtOH were added, and the solution was centrifuged at 12,000 x g for 15 minutes to precipitate the DNA. The precipitated DNA was resuspended in 100µl of 50 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$, 20 units of BamHl and 20 units KpnI and incubated at 37°C for 1 hour. A DNA fragment of the correct size was identified after electrophoresis through an agarose gel and purified using an Ultrafree-MC Filter Unit (Millipore). This fragment was ligated into an E. coli expression vector, pT3XI-2, (described below). Ligation conditions were as follows: the total reaction volume was 5µl and contained 10 ng of pT3XI-2 DNA linearized with KpnI and BamHI, 5 ng of the human GDNF DNA fragment as described above, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1mM DTT, 5% polyethlene glycol-8000, and 1 unit of T4 DNA ligase (Bethesda Research Laboratories). The reaction was incubated at 14°C for 2 hours.

Please replace the paragraph on Page 79, lines 30 to 37 and Page 80, lines 1 to 5 with the following new paragraph:

Next, a XhoI linker purchased from New England Biolabs was inserted into plasmid pCJ1's PvuII site to form plasmid pCJX-1. This insertion disrupts the rop gene which controls plasmid copy number. Next, an EcoRI fragment containing the lacI gene was purified from plasmid pMC9 (Calos et al., 1983), and then inserted into the XhoI site with XhoI to EcoRI adapters. The polylinker region in plasmid pKK223-3 was next replaced with a polylinker containing additional sites by cutting with EcoRI and PstI (SEQ ID NO:24):

5' AATTCCCGGG TACCAGATCT GAGCTCACTA GTCTGCA3'
3' GGGCCC ATGGTCTAGA CTCGAGTGAT CAG 5'

The plasmid vector so obtained is designated pCJXI-1.

Please replace the paragraph on Page 80, lines 32 to 37 and Page 81, lines 1 to 3 with the following new paragraph:

After the 2 hour ligation of the vector with the human mature GDNF construct, 2µl of the reaction mix was used to transform Epicurian Coli SURE® supercompetant E. coli cells (Stratagene) according to the manufacturer's instructions. The DNA sequence of one of the

recombinant plasmids was determined as described in Example 2B. The sequence confirmed that this plasmid contained the complete coding sequence of the human GDNF gene coding for mature human GDNF.

Please replace the paragraph on Page 82, lines 29 to 37 and Page 83, lines 1 to 4 with the following new paragraph:

C. Refolding and bioactivity of recombinant human GDNF produced in bacteria

Preparation of material for refolding. The E. coli transformant JM107 (pT3X12::huGDNF) was grown to stationary phase at 37°C in a yeast extract (#0127-01 Difco Laboratories, Detroit, MI) and tryptone (#0123-05 Difco Laboratories, Detroit, MI) based complex medium (24 g/L yeast extract, 12 g/L tryptone, 5 g/L glycerol, 1.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 ml/L Macol 19/GE60 (4:1), 15 mg/L tetracycline) without IPTG induction. The cells were centrifuged at 16,000xg in a JA10 rotor a 4°C for 20 minutes and the cell paste stored at -20°C.

Please replace the paragraph on Page 84, lines 22 to 34 with the following new paragraph:

Refolding of partially purified TU extract. GDNF, partially purified as described above, was refolded as follows: To 4 ml of pooled column eluate containing GDNF at approximately 1 mg/ml, dithiothreitol was added to 5 mM. The tube was capped so as to exclude air and held at 25°C for 15 minutes. Next, oxidized glutathione disodium salt was added to 15 mM, the tube capped again so as to exclude air, and held at 25°C for 15 minutes. This solution was then diluted with 14 volumes of refold buffer (100 mM Na_2HPO_4, 10 mM ethanolamine, pH 8.3, containing 4 M urea; 5% polyethylene glycol 300; and 2 mM cysteine) and held under argon at 5°C for 3 days.

In the Claims

Prior to examination, please cancel claims 1 to 36, 39 to 40, 42 to 62, and 69 to 74 without prejudice. The claims are currently pending in other applications or will be the subject of additional filings.

Please replace claim 65 with the following:

65. (Amended) The method of claim 63 wherein said cells are cells transformed with a recombinant DNA molecule comprising expression regulatory elements operatively linked to a nucleic acid sequence encoding glial derived neurotrophic factor.

Remarks

A computer disk containing a revised Sequence Listing was submitted on January 16, 1998 for copending application 08/182,183. The revision provides a separate listing (i.e., SEQ ID NO:26) for the amino acid sequence depicted in SEQ ID NO:25. The pre-pro glial cell line-derived neurotrophic factor polypeptide sequence is depicted by amino acid residues 10-220. Please amend the present specification by replacing pages 92-99 of the sequence listing with pages 92-99e as provided.

Also enclosed is an attachment entitled "Version with Markings to Show Changes Made", showing deletions indicated with brackets and additions with double underlining.

Respectfully submitted,

Ron K. Levy
Attorney/Agent for Applicant(s)
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Specification

On page 1, please replace the present title, [GLIAL DERIVED NEUROTROPHIC FACTOR] with GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR.

On page 1, after the title, please insert the following 35 U.S.C. § 120 statement:

CROSS REFERENCE TO RELATED APPLICATION(S)

This application is a divisional of application Serial No. 08/452,229, filed May 26, 1995, which is a which is a divisional of 08/182,183; filed May 23, 1994, which is a 35 U.S.C. §371 filing of PCT/US92/07888, filed September 17, 1992; which is a continuation-in-part of 07/855,413, filed March 19, 1992, now abandoned; which is a part-in-part of 07/788,423, filed November 6, 1991, now abandoned; which is a continuation-in-part of 07/774,109, filed on October 8, 1991, now abandoned; which is a continuation-in-part of 07/764,685, filed September 20, 1991, now abandoned; which are hereby incorporated by reference.

Please replace the paragraph on Page 1, lines 4 to 15 with the following new paragraph:

The present invention relates to neurotrophic factors and glial cell line-derived neurotrophic factor (GDNF) in particular. Also included within this invention are processes for purification of GDNF from natural sources and processes for cloning rat and human genes encoding GDNF, as well as the nucleic acid sequence of the rat and human genes that encode GDNF. The GDNF gene has been subcloned into an expression vector, and the vector used to express biologically active GDNF. In addition, this invention includes the use of GDNF for preventing and treating nerve damage and nerve related diseases such as Parkinson's disease.

Please replace the paragraph on Page 7, lines 21 to 28 with the following new paragraph:

This invention relates to and claims substantially purified glial cell line-derived neurotrophic factor (GDNF). In one embodiment of this invention, substantially purified GDNF is obtained having a specific activity at least about 24,000 times greater than the specific activity of B49 conditioned medium. The substantially purified GDNF has a specific activity of at least about 12,000 TU/μg.

Please replace the paragraph on Page 8, lines 1 to 7 with the following new paragraph:
The amino acid sequence of mature and "pre-pro" forms of rat GDNF is as set forth in Figs. 13 and 14 (SEQ ID NO:3 and SEQ ID NO:4). The amino acid sequence of mature human GDNF is as set forth in the underlined portion of Fig. 19 [(SEQ ID NO:5)] (SEQ ID NO:6). The amino acid sequence of the pre-pro form of human GDNF is set forth in Figures 19 (SEQ ID NO:5) and 22 (SEQ ID NO:8).

Please replace the paragraph on Page 9, lines 28 to 37 and Page 10, lines1 to 2 with the following new paragraph:

This invention includes substantially purified antibodies that recognize GDNF. Also included is a method for preventing or treating nerve damage which comprises implanting cells that secrete glial cell line-derived neurotrophic factor into the body of patients in need thereof. Finally, the present invention includes a device for preventing or treating nerve damage by implantation into a patient comprising a semipermeable membrane, and a cell that secretes GDNF encapsulated within said membrane and said membrane being permeable to GDNF and impermeable to factors from the patient detrimental to the cells.

Please replace the paragraph on Page 11, lines 5 to 12 with the following new paragraph:

Figure 8 (SEQ ID NO:1) describes the amino-terminal amino acid sequence obtained from purified GDNF. The empty parenthesis indicates a position where the amino acid could not be determined using the sequencing technique employed. Where residues are given in parentheses, there was some uncertainty as to the identification of that residue. The complete correct amino-terminal amino acid sequence is shown in Figure 19 (SEQ ID NO: 4) below.

Please replace the paragraph on Page 11, lines 25 to 26 with the following new paragraph:
Figure 12 (SEQ ID NO:2) describes an internal amino acid sequence obtained from purified GDNF.

Please replace the paragraph on Page 11, lines 28 to 34 with the following new paragraph:
Figure 13 (SEQ ID NO:3) depicts the nucleic acid sequence obtained for rat GDNF derived from a B49 cell library cDNA clone AzapII76.1. [Also depicted is] SEQ ID NO:4 depicts the inferred amino acid sequence for rat GDNF. [The] In Figure 13, the nucleic acid sequence coding for

mature GDNF is underlined. The amino-terminal sequence of the most preferred pre-pro form of GDNF is marked with an *.

Please replace the paragraph on Page 11, lines 36 to 37 with the following new paragraph:

Figure 14 (SEQ ID NO:4) depicts the inferred amino acid sequence of mature rat GDNF.

Please replace the paragraph on Page 13, lines 17 to 23 with the following new paragraph:

Figure 19 (SEQ ID NO:5) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2C below, including the entire portion of the gene encoding for mature human GDNF. [Also depicted is] SEQ ID NO:6 depicts the inferred amino acid sequence for mature human GDNF. [The] In Figure 19, the amino acid sequence for mature human GDNF is underlined.

Please replace the paragraph on Page 14, lines 18 to 27 with the following new paragraph:

Figure 22 (SEQ ID NO:8) depicts a portion of the nucleic acid sequence obtained for human GDNF, as described in Example 2D below, including the coding sequence of amino acids of 1-50 of human pre-pro GDNF. Also depicted is the inferred amino acid sequence for the first 50 amino acids of human pre-pro GDNF. This information, in conjunction with coding sequence information given in Figure 19, provides the full coding sequence for human pre-pro GDNF, and the inferred amino acid sequence for the human pre-pro GDNF protein.

Please replace the paragraph on Page 16, lines 14 to 24 with the following new paragraph:

Prior to this invention, GDNF had not been identified as a discrete biologically active substance [or] and had not existed in a substantially pure form. As described herein, a detailed description of GDNF is provided, along with a description of: its physical, chemical and biological characteristics; its utility; how to make it; useful compositions containing it; nucleic acid sequences coding for it; vectors containing such nucleic acid sequences; host cells transformed by such vector; recombinant techniques for its production; and other aspects of the invention.

Please replace the paragraph on Page 18, lines 29 to 33 with the following new paragraph:

Probes were prepared based on the nucleic acid sequence of rat GDNF in order to clone the genomic DNA gene coding for human GDNF. The human gene encoding mature GDNF, and the amino acid sequence of human mature GDNF are given in Figure 19 [(SEQ ID NO:5)] (SEQ ID NOS:5 and 6, respectively).

Please replace the paragraph on Page 19, lines 17 to 26 with the following new paragraph:

Throughout this specification, any reference to glial cell line-derived neurotrophic factor should be construed to refer to neurotrophic factors of any origin which are substantially homologous to and which are biologically equivalent to the GDNF characterized and described herein. The degree of homology between the rat and human protein is about 93% and all mammalian GDNF will have a similarly high degree of homology. Such GDNFs may exist as dimers in their biologically active form.

Please insert the following paragraph on Page 20, line 22 after "reference.":

In particular, Dayhoff describes that "[i]n practice, two related proteins may be aligned with the insertion of an average of 3 or 4 gaps in a length of 100 residues. About 20% of the aligned amino acids are identical. Under these conditions, the statistical conclusion of common ancestry can be drawn with great confidence. Common ancestry may exist even though it cannot be proved from the comparison of two sequences. The use of additional evidence, such as the correspondence of the active sites, the comparisons of many related sequences with one new one, and the nature of the three-dimensional structures, will eventually permit the inference of relationships of even more remotely related structures."

Please replace the paragraph on Page 26, lines 11 to 20 with the following new paragraph:

The procedure for obtaining the nucleotide sequence of the cDNA clone contained in λZapII76.1 is given in Example 2B. below. The nucleotide sequence of the first 877 base pairs of the 5' end of the cDNA clone was determined, and is shown in Figure 13 [(SEQ ID NO:2)] (SEQ ID NO:3). In Figure 13, the clone shown contains an open reading frame (ORF) of 227 amino acids that includes the amino-terminus of purified GDNF and is consistent with the sequence for an internal peptide obtained by cleavage of purified GDNF.

Please replace the paragraph on Page 26, lines 21 to 38 and Page 27, lines 1 to 9 with the following new paragraph:

The inferred amino acid sequence given in Figure 14 (SEQ ID NO:4) shows the amino acid sequence for the "mature GDNF". By "mature GDNF", is meant the sequence of the purified GDNF obtained from the B49 conditioned medium. Of course, the purified GDNF may exist as a dimer or other multimer and may be glycosylated or chemically modified in other ways. Mature GDNF may be truncated at the carboxyl terminus, in particular by proteolytic processing of the lys-arg residues 6 and 5 residues from the carboxyl terminal end. Examination of the nucleic acid sequence of the λZapII76.1 rat clone as shown in Fig. 13 [(SEQ ID NO:2)] (SEQ ID NO:3) suggests that GDNF is initially translated as pre-pro-GDNF polypeptide and that proteolytic processing of the signal sequence and the "pro" portion of this molecule result in purified GDNF having the same mature sequence as that obtained from B49 conditioned medium. It is postulated, that the pre-pro-GDNF polypeptide begins at the first ATG -- methionine encoding-- codon at the 5' end of the clone (position 50 in Figure 13). The present invention includes, therefore, any and all pre-pro GDNF polypeptides that may be translated from the gene shown in Figure 13, as well as any and all pre-pro GDNF polypeptides translated from a more complete clone that may be easily obtained by one of skill in the art using standard laboratory procedures and the clone described herein.

Please replace the paragraph on Page 27, lines 10 to 24 with the following new paragraph:

Review of the rat nucleic acid sequence given in Fig. 13 [(SEQ ID NO:2)] (SEQ ID NO:3) shows that the predicted amino acid sequence located between positions 518 and 538 is Asp-Lys-Ile-Leu-Lys-Asn-Leu which is consistent with the amino acid sequence determined for a peptide derived from purified mature GDNF by the process described in the section on internal sequence in Example 1 below. A TGA stop codon at position 706 terminates the ORF. The predicted length of the purified GDNF is thus 134 amino acid residues, and the predicted molecular weight of this polypeptide is 14,931. Two potential N-linked glycosylation sites occur at positions 425 and 533. Glycosylation at either or both of these sites would increase the molecular weight of the molecule.

Please replace the paragraph on Page 30, lines 11 to 27 with the following new paragraph:

Specific nucleic acid sequences can be modified by those of skill in the art. Therefore, this invention also includes all nucleic acid sequences which encode for the amino acid sequences for mature rat and mature human GDNF as set forth in Figures 14 (SEQ ID NO:4) and 19 [(SEQ ID NO:5)] (SEQ ID NO:6), and pre-pro rat GDNF as set forth in Figure 13 (SEQ ID NO:3) and for pre-pro human GDNF as set forth in Figures 19 (SEQ ID NO:5) and 22 (SEQ ID NO:8). The present invention also incorporates nucleic acid sequences which will hybridize with all such nucleic acid sequences -- or the [compliments] complements of the nucleic acid sequences where appropriate -- and encode for a polypeptide having dopaminergic activity. The present invention also includes nucleic acid sequences which encode for polypeptides that have dopaminergic activity and that are recognized by antibodies that bind to GDNF.

Please replace the paragraph on Page 36, lines 23 to 37 and Page 37, lines 1 to 16 with the following new paragraph:

An alternative method for identifying GDNF family members involves use of the polymerase chain reaction (PCR) to amplify sequences from GDNF family members followed by cloning and analysis of amplified sequences. Degenerate (or nondegenerate) oligonucleotide primers for PCR may be synthesized based on the sequence of GDNF. Given the conservation of cysteine location and the conservation of amino acid sequences in the immediate vicinity for the cysteine residues that is observed for the NGF family, the regions around the cysteines in mature GDNF represent obvious candidates for primer synthesis but a variety of other primers could also be chosen from both the mature and pre-pro-portions of the protein. PCR reactions may be performed under conditions of reduced annealing temperature which would allow amplification of not only the GDNF sequence but the sequences of any GDNF family members. See, Innis et al. 1990 PCR [Protocals:] Protocols: A Guide to Methods and Applications, Academic Press. The products of such PCR reactions may be size selected by gel electrophoresis, cloned into an appropriate vector and the cloned DNA sequenced to identify GDNF family members. Alternatively, the clones may first be screened by hybridization to a probe specific for GDNF under conditions of high stringency to identify GDNF clones. Any clones that fail to hybridize to GDNF under high stringency would then be sequenced or such clones could be hybridized to a GDNF probe under conditions of reduced stringency and any clone that did hybridize to the GDNF probe under these conditions would then be sequenced.

Please replace the paragraph on Page 45, lines 25 and 37 to Page 46, lines 1 to 9 with the following new paragraph:

The functional status of dopaminergic neurons may be assayed in these cultures by measuring dopamine uptake through specific "scavenger" transporters in the dopaminergic neurons and by counting the number of neurons positive for the dopamine synthetic enzyme tyrosine hydroxylase using immunohistochemistry. The possibility of significant contamination of the cultures with the noradrenergic neurons, which can also transport dopamine and also contain tyrosine hydroxylase, was ruled out by careful dissection and by demonstrating that the dopamine transporters have the pharmacological profile characteristic of [dompainergic] dopaminergic, rather than noradrenergic, neurons. Dopamine uptake in these cultures is inhibited by GBR12909, an inhibitor of the monoamine transporter on [dompainergic] dopaminergic neurons, with an ED_{50} of 20nM. In contrast, at least 300-fold more desipramine, an inhibitor of monoamine transporter or noradrenergic neurons, is required to inhibit dopamine uptake in their cultures. These values are those that have been reported for the monoamine transporter in dopaminergic neurons.

Please replace the paragraph on Page 51, lines 1 to 37 and Page 52, lines 1 to 3 with the following new paragraph:

Internal sequence: GDNF preparation after step 3 of the purification described above was used as the starting material to obtain internal sequence. Fractions 5 and 6 in Figure 3 were pooled into a siliconized microfuge tube containing 9 µl of 0.4% Tween and concentrated to 40 µl on a speed vac. Added to the sample were 160 µl of 1% NH_4HCO_3 containing 2.5 M guanidine hydrochloride and 1 µg of trypsin, and the sample was incubated overnight at 37°C. The mixture was acidified with 20 µl of 25% TFA, concentrated to about 150 µl on a speed vac, and peptides were separated on a narrow bore Aquapore RP-300 C8 reverse phase HPLC column (Brownlee column), 2.1 x 220 mm, and eluted with an H_2O/0.1% TFA:80% acetonitrile/0.085% TFA gradient. Peptide containing fractions were collected manually into siliconized microfuge tubes based on the absorption at 214 nm. Figure 9 shows the results of such chromatography. Sequence of peak 10 in Figure 9 was determined to be identical to the first 13 amino acid residues of the amino-terminal sequence of the untreated protein shown in Figure 8 (SEQ ID NO:1). Peak 37 in Figure 9 was further treated with CNBr. The sample was concentrated to 20 µl on a speed vac. Added to the sample was 70 µl of 90% formic acid and 5 mg of CNBr, and

the sample was incubated in the dark overnight at room temperature. This mixture was concentrated to 20 µl on a speed vac, diluted with 100 µl of 0.1% TFA and separated on reverse phase HPLC as described above. Figure 10 shows the results of such chromatography. Peak 1 in Figure 10 was concentrated to 20 µl in the presence of 5 µl of 0.4% Tween 20 on a speed vac. Added to the sample was 100 µl of 1% NH_4HCO_3 and 5 µl of 50 mM dithiothreitol and the sample was incubated at room temperature for an hour. The mixture was acidified with 10 µl of 25% TFA, concentrated to 100 µl on a speed vac and separated on reverse phase HPLC as above. Figure 11 shows the results of such chromatography. Both peaks 33 and 34 in Figure 11 gave an identical sequence (Figure 12) (SEQ ID NO:2).

Please replace the paragraph on Page 64, lines 27 to 37 and Page 65, lines 1 to 11 with the following new paragraph:

Six λFIX II [clones] clones from a human genomic library were identified by hybridization with a rat GDNF probe and plaque-purified to homogeneity (see above). Lysates of each phage were prepared by the method of Sambrook et al. (Molecular Cloning: A Laboratory Manual; 1989). DNA was prepared from these clones by the following procedure: DNAase I (Pharmacia) and RNAase A (Sigma) was added to 5 ml of each culture to give a final concentration of 1 µg/ml. The solution was incubated at 37°C for 1 hour. Then 5 ml of 20% polyethylene glycol (Sigma), 2M NaCl, was added and the solution was incubated at 0°C for 1 hour. The [λphage] λphages were pelleted by centrifugation at 12,000 x g for 10 min. The phage pellet was resuspended in 250 µl of TE (10mM TRIS, pH 7.4, 1mM EDTA) and sequentially extracted with an equal volume of: a. chloroform; b. phenol; c. a 1:1 mixture of chloroform and phenol; and d. chloroform. Ammonium acetate was added to give a final concentration of .25 M and the DNA was precipitated by the addition of 2 volumes of ethanol and centrifugation at 10,000 x g. The DNA pellet was resuspended in TE.

Please replace the paragraph on Page 66, lines 35 to 37 and Page 67, lines 1 to 10 with the following new paragraph:

To obtain the complete human pre-proGDNF sequence, a radiolabeled hybridization probe may be made based on the sequence of human GDNF already [obtained and use this to screen human cDNA] obtained, and this probe may be used to screen human cDNA libraries. Because cDNAs are copies of the processed mRNA, the introns are not present and the sequence

of the complete coding sequence can be obtained. Alternatively, now that the position of the intron relative to the coding sequence in known, a hybridization probe that is specific for sequences upstream of the intron can be made from the rat DNA clone and this probe can be used to screen a genomic library for clones that contain the 5' exon(s).

Please replace the paragraph on Page 67, lines 12 to 23 with the following new paragraph:

D. Nucleotide Sequence Encoding the First 50 Amino Acids of Human Pre-proGDNF

As detailed in Example 2C, there is an intron that splits the nucleotide sequence corresponding to amino acid 51 of human pre-proGDNF (i.e. between the T and CA forming the codon for the serine residue at position 51). In order to obtain the sequence of this portion of the molecule, a human genomic library was screened with a probe derived from the amino-terminal coding sequence of rat pre-proGDNF and one hybridizing clone was sequenced and shown to contain the coding sequence of amino acids 1 through 50 of human pre-proGDNF as shown in Figure 22 (SEQ ID NO:8). SEQ ID NO:25 and SEQ ID NO:26 present nucleotide and amino acid sequences, respectively, for a composite pre-pro sequence as depicted in Figures 22 and 19 as well as SEQ ID NOS:8 and 5. A pre-pro form of human glial cell line-derived neurotrophic factor polypeptide is set forth in SEQ ID NO:26 amino acid residues 10 through 220.

Please replace the paragraph on Page 68, lines 13 to 37 and Page 69, lines 1 to 4 with the following new paragraph:

The cloned DNAs of these recombinant phages were analyzed by Southern blot hybridization. An approximately 1000 bp AluI fragment was found to hybridize to the screening probe and was subcloned into SmaI-digested pBluescript SK- to produce pBSSK-λ3AluI. This cloned AluI fragment was further subcloned to facilitate sequencing of the relevant DNA segment. Purified pBSSK-λ3AluI DNA was digested with a series of restriction enzymes that cleave the vector only once (within the polylinker region) and the digestion products were analyzed by agarose gel electrophoresis. Two restriction enzymes (PstI and SacII) were thus identified that cleaved once within the cloned DNA. Figure 22 shows a map of SacII and PstI sites. Southern blots revealed that the region of the cloned segment that hybridized to the screening probe was located between the SacII and PstI sites of the cloned AluI segment. Therefore, for sequencing, two deletion derivatives of pBBSK- λ3AluI were constructed. In one instance the small, about 300 bp, PstI fragment was deleted as follows: The plasmid was

digested with PstI and the digest was ligated and transformed into E. coli. Transformants were screened for those lacking the small PstI fragment. In parallel, the 300 bp SacII fragment was similarly deleted from pBSSK-λ3AluI to produce a second deletion derivative. These two deletion plasmids were used as templates for sequencing reactions. Sequencing was carried out as described in Example 2B.

Please replace the paragraph on Page 78, lines 16 to 37 and Page 79, lines 1 to 10 with the following new paragraph:

The reaction conditions for the amplification of the human GDNF are as follows: the total reaction volume was 100µl and contained 1 ng of a λDNA clone containing the human GDNF gene, 20 pmoles each of PD3 and PD4, 20 mM Tris-HCl pH8.8, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, and 0.1% Triton X-100. The reaction mixture was heated to 95°C for 5 minutes, cooled to 44°C, and 2 units of Pfu DNA polymerase (Stratagene) [was] were added. The reaction consisted of 30 cycles of: 72°C for 1½ minutes, 95°C for 1 minute, and 44°C for 1½ minutes. At the end of the reaction, $MgCl_2$ was added to a final concentration of 10 mM. 5 units of DNA polymerase I large (Klenow) fragment (Promega) [was added] were added, and the reaction incubated at 37°C for 10 minutes. Then 10µl of 3M NaAc and 220µl of EtOH [was added] were added, and the solution was centrifuged at 12,000 x g for 15 minutes to precipitate the DNA. The precipitated DNA was resuspended in 100µl of 50 mM Tris-HCl (pH 8), 50 mM NaCl, 10 mM $MgCl_2$, 20 units of BamHl and 20 units KpnI and incubated at 37°C for 1 hour. A DNA fragment of the correct size was identified after electrophoresis through an agarose gel and purified using an Ultrafree-MC Filter Unit (Millipore). This fragment was ligated into an E. coli expression vector, pT3XI-2, (described below). Ligation conditions were as follows: the total reaction volume was 5µl and contained 10 ng of pT3XI-2 DNA linearized with KpnI and BamHI, 5 ng of the human GDNF DNA fragment as described above, 50 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 1 mM ATP, 1mM DTT, 5% polyethlene glycol-8000, and 1 unit of T4 DNA ligase (Bethesda Research Laboratories). The reaction was incubated at 14°C for 2 hours.

Please replace the paragraph on Page 79, lines 30 to 37 and Page 80, lines 1 to 5 with the following new paragraph:

Next, a XhoI linker purchased from New England Biolabs was inserted into plasmid pCJ1's PvuII site to form plasmid pCJX-1. This insertion disrupts the rop gene which controls plasmid copy number. Next, an EcoRI fragment containing the lacI gene was purified from plasmid pMC9 (Calos et al., 1983), and then inserted into the XhoI site with XhoI to EcoRI adapters. The polylinker region in plasmid pKK223-3 was next replaced with a polylinker containing additional sites by cutting with EcoRI and PstI (SEQ ID NO:24):

5' AATTCCCGGG TACCAGATCT GAGCTCACTA GTCTGCA3'
3' GGGCCC ATGGTCTAGA CTCGAGTGAT CAG 5'

The plasmid vector so obtained is designated pCJXI-1.

Please replace the paragraph on Page 80, lines 32 to 37 and Page 81, lines 1 to 3 with the following new paragraph:

After the 2 hour ligation of the vector with the human mature GDNF construct, 2µl of the reaction mix was used to transform Epicurian Coli SURE® supercompetant E. coli cells [(Statagene)] (Stratagene) according to the manufacturer's instructions. The DNA sequence of one of the recombinant plasmids was determined as described in Example 2B. The sequence confirmed that this plasmid contained the complete coding sequence of the human GDNF gene coding for mature human GDNF.

Please replace the paragraph on Page 82, lines 29 to 37 and Page 83, lines 1 to 4 with the following new paragraph:

C. Refolding and bioactivity of recombinant human GDNF produced in bacteria

Preparation of material for refolding. The E. coli transformant JM107 (pT3X12::huGDNF) was grown to stationary phase at 37°C in a yeast extract (#0127-01 Difco Laboratories, Detroit, MI) and tryptone (#0123-05 Difco Laboratories, Detroit, MI) based complex medium (24 g/L yeast extract, 12 g/L tryptone, 5 g/L glycerol, 1.3 g/L K_2HPO_4, 0.4 g/L KH_2PO_4, 0.1 ml/L Macol 19/GE60 (4:1), 15 mg/L tetracycline) without IPTG induction. The

cells were centrifuged at 16,000xg in a JA10 rotor a 4°C for 20 minutes and the cell paste stored at [- 20°C] -20°C.

Please replace the paragraph on Page 84, lines 22 to 34 with the following new paragraph:

Refolding of partially purified TU extract. GDNF, partially purified as described above, was refolded as follows: To 4 ml of pooled column eluate containing GDNF at approximately 1 mg/ml, dithiothreitol was added to 5 mM. The tube was capped so as to exclude air and held at 25°C for 15 minutes. Next, oxidized glutathione disodium salt was added to 15 mM, the tube capped again so as to exclude air, and held at 25°C for 15 minutes. This solution was then diluted with 14 volumes of refold buffer [(100 mM Na_2HPO_4)] (100 mM Na_2HPO_4, 10 mM ethanolamine, pH 8.3, containing 4 M urea; 5% polyethylene glycol 300; and 2 mM cysteine) and held under argon at 5°C for 3 days.

In the Claims

Prior to examination, please cancel claims 1 to 36, 39 to 40, 42 to 62, and 69 to 74.

Please replace claim 65 with the following:

65. (Amended) The method of claim 63 wherein said cells are [the cells of claim 42] transformed with a recombinant DNA molecule comprising expression regulatory elements operatively linked to a nucleic acid sequence encoding glial derived neurotrophic factor.